

Normal and tumor-derived myoepithelial cells differ in their ability to interact with luminal breast epithelial cells for polarity and basement membrane deposition

Thorarinn Gudjonsson¹, Lone Rønnov-Jessen², René Villadsen¹, Fritz Rank³, Mina J. Bissell⁴ and Ole William Petersen^{1,*}

¹Structural Cell Biology Unit, Institute of Medical Anatomy, The Panum Institute, DK-2200 Copenhagen N, Denmark

²Zoophysiological Laboratory, The August Krogh Institute, DK-2100 Copenhagen Ø, Denmark

³Department of Pathology, Rigshospitalet, DK-2100 Copenhagen Ø, Denmark

⁴Life Sciences Division, Berkeley National Laboratory, Berkeley, CA 94720, USA

*Author for correspondence (e-mail: o.w.petersen@mai.ku.dk)

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Summary

The signals that determine the correct polarity of breast epithelial structures *in vivo* are not understood. We have shown previously that luminal epithelial cells can be polarized when cultured within a reconstituted basement membrane gel. We reasoned that such cues *in vivo* may be given by myoepithelial cells. Accordingly, we used an assay where luminal epithelial cells are incorrectly polarized to test this hypothesis. We show that culturing human primary luminal epithelial cells within collagen-I gels leads to formation of structures with no lumina and with reverse polarity as judged by dual stainings for sialomucin, epithelial specific antigen or occludin. No basement membrane is deposited, and $\beta 4$ -integrin staining is negative. Addition of purified human myoepithelial cells isolated from normal glands corrects the inverse polarity, and leads to formation of double-layered acini with central lumina. Among the laminins present in the human breast basement membrane (laminin-1, -5 and -10/11), laminin-1 was unique in its ability to substitute for myoepithelial cells in polarity reversal.

Myoepithelial cells were purified also from four different breast cancer sources including a biphasic cell line. Three out of four samples either totally lacked the ability to interact with luminal epithelial cells, or conveyed only

correction of polarity in a fraction of acini. This behavior was directly related to the ability of the tumor myoepithelial cells to produce α -1 chain of laminin. *In vivo*, breast carcinomas were either negative for laminin-1 (7/12 biopsies) or showed a focal, fragmented deposition of a less intensely stained basement membrane (5/12 biopsies). Dual staining with myoepithelial markers revealed that tumor-associated myoepithelial cells were either negative or weakly positive for expression of laminin-1, establishing a strong correlation between loss of laminin-1 and breast cancer. We conclude that the double-layered breast acinus may be recapitulated in culture and that one reason for the ability of myoepithelial cells to induce polarity is because they are the only source of laminin-1 in the breast *in vivo*. A further conclusion is that a majority of tumor-derived/-associated myoepithelial cells are deficient in their ability to impart polarity because they have lost their ability to synthesize sufficient or functional laminin-1. These results have important implications for the role of myoepithelial cells in maintenance of polarity in normal breast and how they may function as structural tumor suppressors.

Key words: Breast cancer, Morphogenesis, Laminin, Reversion, Polarity, Myoepithelial function

Introduction

The normal human breast gland comprises a branching ductal-lobular system lined by an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells separated from the interstitial stroma by an intact basement membrane. The luminal epithelial cells are polarized glandular cells with specialized apical and basolateral membrane domains expressing sialomucin and adhesion molecules, respectively (Rønnov-Jessen et al., 1996). The myoepithelial cells contribute significantly to the formation of basement membrane, and their myogenic differentiation is responsible for the contractile phenotype mediated by oxytocin (Murrell, 1995). Because breast cancer arises mainly in the luminal epithelial compartment, until recently little attention was paid to the role of the surrounding cells in breast function. A number

of laboratories including ours have postulated that intact myoepithelial cells are an important determinant of normal breast differentiation, and that loss of their functions could contribute to induction and/or progression of epithelial cancer (Zou et al., 1994; Radice et al., 1997; Sternlicht et al., 1997; Pécoux et al., 1999; Slade et al., 1999). Indeed a number of myoepithelial-specific proteins have been shown to inhibit epithelial tumor formation and, thus, these cells are postulated to have tumor suppressive activities (Gomm et al., 1997; Sternlicht et al., 1997; Shao et al., 1998; Sun et al., 1999). The tumor-suppressive proteins include α -smooth muscle actin (Tobacman, 1997; Okamoto-Inoue et al., 1999), cytokeratin 5 (Zajchowski et al., 1990), $\alpha 6$ integrin (Sager et al., 1993), caveolin-1 (Lee et al., 1998), connexin 43 (Hirschi et al., 1996), maspin (Zou et al., 1994), TIMP-1 (Sternlicht et al., 1997),

myoepithelium-derived serine proteinase inhibitor (MEPI) (Xiao et al., 1999), relaxin (Bani et al., 1994) and activin (Liu et al., 1996).

Whereas these data provide correlative support for myoepithelial-specific proteins to act as tumor suppressors when overexpressed in luminal epithelial cells, there is little experimental data to show how myoepithelial cells themselves could fulfill such a function. In addition, it is not known whether myoepithelial cells change function with the process of tumor formation (Wetzels et al., 1989; Malzahn et al., 1998). Since there were no assays to assess the functional activities of myoepithelial cells, we set out first to establish an assay, and second to investigate whether normal and tumor-derived myoepithelial cells behave differently in such assays. We show that normal myoepithelial cells can re-establish polarity in luminal epithelial cells in 3D collagen-I gels leading to formation of double-layered acini. We further show that even when tumor-derived myoepithelial cells retain myoepithelial characteristics, they differ from those derived from non-malignant cells in this assay. Finally, we show that the functional difference between non-malignant and tumor-derived myoepithelial cells appears to be the production of basement membrane protein laminin-1.

Materials and Methods

Cell culture

Breast luminal epithelial cells and myoepithelial cells were generated from primary cultures of biopsies from patients undergoing reduction mammoplasty for cosmetic reasons, and from residual tissue from mastectomy specimens with breast carcinoma. The cancer tissue was removed from the tumors by a trained pathologist. The tissue was prepared as previously described (Péchoux et al., 1999). Briefly, it was mechanically disaggregated followed by enzymatic disaggregation with collagenase to release epithelial organoids. The organoids were plated in CDM3 medium (Petersen and van Deurs, 1987) in collagen-coated (Vitrogen-100, Palo Alto, CA) T-25 flasks (Nunc, Roskilde, Denmark). Cells were kept at 37°C in a humidified incubator and the medium was changed three times a week.

Cell separation and cell lines

Luminal epithelial cells and myoepithelial cells were separated from each other after organoids had spread out to monolayers in primary culture. Cells were trypsinized and filtered as previously described (Péchoux et al., 1999). Luminal epithelial cell preparations that were more than 99% pure were generated by use of immunomagnetically retained cells in a column from two independent separations with anti-sialomucin antibody (115D8, kindly provided by J. Hilgers, The Netherlands) and the myoepithelial cells were collected from the flow-through. All cell separations were carried out by use of the MiniMACS magnetic cell separation system according to the manufacturer's instructions (Miltenyi Biotec, GmbH, Gladbach, Germany, purchased from Biotech Line, Lyngby, Denmark). To obtain up to 100% pure populations, cell suspensions were passed through columns at the highest possible flow rate (i.e. with no needles attached to the column). The purified cells were plated on collagen-coated flasks in either CDM3 medium (luminal epithelial cells) or Dulbecco's modified Eagle's medium-Ham's F12 (DME-F12) supplemented with cholera toxin and epidermal growth factor (myoepithelial cells) (Petersen and van Deurs, 1988). Myoepithelial cells from primary carcinomas were purified by retaining cells in an anti-Thy-1 column (ASO-2, Dianova, GmbH). The sorted cells were used directly for co-culture experiments. For some experiments we used a myoepithelial

cell line purified by anti-Thy-1 (ASO2,) and immortalized by transfection with a retroviral construct (LXSN; ATCC) containing the HPV16 E6 and E7 oncogenes (Band et al., 1990) as described previously (Péchoux et al., 1999). As control cells we used purified fibroblasts from reduction mammoplasties (Rønnov-Jessen and Petersen, 1993), a human osteosarcoma cell line, Saos-2 (Clontech, Palo Alto, CA) and HMT-3909S16 (see below).

The cancer myoepithelial cell line, HMT-3909S16, was purified from HMT-3909S13 (Petersen et al., 1990) by first collecting floating cells after plating had taken place for the majority of the cells, and transferring these to another culture flask. Next, the myoepithelial cells were purified by immunomagnetic sorting (see above) with anti- α 1-integrin (VLA-1, T Cell Diagnostics, Cambridge, MA) as retaining antibody since α 1-integrin has been shown to be specific for myoepithelial cells (Rønnov-Jessen et al., 1996). The subline has now been cultured for 63 passages.

Clonal cultures of HMT-3909S13 were obtained by plating single cells at low density in T-25 flasks.

Collagen-I gel and rBM experiments

For 3D cultures, 2×10^5 luminal epithelial cells were plated inside hydrated collagen-I gel (Vitrogen100) or a reconstituted basement membrane (rBM; Matrigel®, lot# 40230A, Collaborative Biomedical Products, Bedford, MA). In some experiments rBM was added to a final concentration of 10% (volume) to the collagen I gels. The collagen-I gels were prepared according to the manufacturer's instructions. Experiments were carried out in 24-well dishes using 500 μ l collagen-I gel or 300 μ l rBM and single cell suspensions. Whenever co-culture experiments were performed, the interacting cells were premixed in equal numbers in suspension prior to embedding in gels. All experiments were carried out in CDM3 media or CDM3 supplemented with 20% FCS. In replacement experiments instead of myoepithelial cells in the collagen-I assay, we used 10 or 100 μ g/ml laminin-1 (Sigma-Aldrich A/S, Vallensbæk, Denmark), 10 or 100 μ g/ml laminin 10/11 (affinity purified from human placenta with mAb 4C7) (Ferletta and Ekblom, 1999) (cat. #12163-010; Gibco BRL, Life Technologies A/S, Tåstrup, Denmark), or 2.5 μ g/ml affinity purified laminin-5 (kindly provided by Vito Quaranta, The Scripps Research Institute, La Jolla, CA and Jonathan Jones, Department of Pediatrics, Northwestern University Medical School, Chicago, IL) (Koshikawa et al., 2000) or 5 μ g/ml LG3 module of laminin-5 (Shang et al., 2001) (kindly provided by Vito Quaranta).

Immunocytochemistry

For characterization of interacting cells, normal breast, breast carcinoma biopsies, and the gels were frozen in n-hexan and mounted in Tissue Freezing Medium™ (Leica Instruments, Heidelberg, GmbH) for sectioning. Routinely, we used 8 μ m sections that were air dried for 15 minutes and fixed in methanol at -20°C. The sections were preincubated with PBS supplemented with 10% goat serum. This buffer was also used for dilution of antibodies and for rinsing. Primary antibodies included anti-sialomucin (MAM6, clone 115D8), anti-occludin (OC-3F10, Zymed Laboratories, San Francisco, CA), anti-epithelial-specific antigen (ESA; VU-1D9, NovoCastra, Newcastle upon Tyne, UK) (Stingl et al., 1998), anti-Thy-1 (ASO-2, Dianova), anti-cytokeratin (CAM5.2, Becton Dickinson, Mountain View, CA), anti-smooth muscle actin (HHF-35, ENZO Diagnostics, New York, NY), anti-vimentin (V9, DAKO, Glostrup, Denmark), anti- α 1 chain, laminin-1 (EB7, kindly provided by I. Virtanen, University of Helsinki), anti- α 3 chain, laminin 5 (BM-165, kindly provided by R. E. Burgeson, Harvard Medical School, MA), anti- α 5 chain, laminin 10/11 (4C7, DAKO), anti- β 4-integrin (3E1, Chemicon International, Temecula, CA), anti-maspin (clone 7, Transduction Laboratories, Lexington, KY), anti-keratin K17 (M7046, DAKO), anti-keratin-associated protein (BG3C8 (Pallesen et al., 1987), kindly

provided by J. E. Celis, The Danish Cancer Society, Denmark) and anti-type IV collagen (CIV 22, DAKO). Immunoperoxidase staining was performed as previously described using rabbit anti-mouse immunoglobulins as secondary antibody (Z259, DAKO) and PAP complex as tertiary antibody (P850, DAKO) (Petersen and van Deurs, 1988). For immunofluorescence and double-labeling experiments we used iso-type specific antibodies all from Southern Biotechnology (Birmingham, AL) as previously described (Rønnov-Jessen et al., 1992). Antibody incubations were carried out for 30 minutes while rinses were done twice, for 5 minutes each, all at room temperature. Some sections received a nuclear counter stain with 1 µg/ml propidium iodide (Molecular Probes, Oregon, USA). Afterwards sections were mounted with coverslips by use of Fluoromount-G (Southern Biotechnology) supplemented with 2.5 mg/ml n-propyl gallate (Sigma-Aldrich) as previously described (Rønnov-Jessen et al., 1992). Immunofluorescence was visualized using a Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Jena GmbH). Sections were observed by use of a 20×, 40× or 63× objective and sliced in the z-plane in 0.25 µm intervals and exposed to visualize FITC and Texas red or propidium iodide. Polarization was quantified by counting all acinar profiles (approximately 100) in three different cryostat sections of collagen-I gels from five different biopsies, all stained with immunoperoxidase against sialomucin and counterstained with hematoxylin. Polarization was described as fractional when the frequency of correctly polarized profiles was lower than that obtained in normal biopsies.

RNA isolation and RT-PCR

Luminal epithelial cells embedded in rBM or collagen-I gel as well as monolayer cultures of primary purified myoepithelial cells, MCF-7 S9 (Briand and Lykkesfeldt, 1986), HMT-3909S16 and immortalized myoepithelial cells were harvested using TRIzol reagent, and total RNA was extracted according to the manufacturer's instructions (Life Technologies, Roskilde, Denmark).

1.3 µg DNase-treated total RNA (DNase I Amp Grade, Life Technologies) was used as template for first strand synthesis with oligo dT primers (SuperScript Preamplification System, Life Technologies) in a 30 µl volume. A volume of 1 µl from this cDNA served as template for the subsequent PCR-amplifications, using primers specific for integrin-β4 (ITGB4), laminin subunit α1, α3 and α5 (LAMA1, LAMA3 and LAMA5, respectively), collagen type IV α1 and α2 (COL4A1 and COL4A2, respectively), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). The primers were LAMA1-FW: 5'-AAAGTCGCCGTGTCTGCAGAC-3', LAMA1-RV: 5'-TTAAATGAGTAACCTTCACAGC-3' (Engvall et al., 1992; Slade et al., 1999), LAMA3-FW: 5'-AGCTCTTGCTGAACCGGATA-3', LAMA3-RV: 5'-AATGGCTCCCAAAGC-TCTCT-3', LAMA5-FW: 5'-ACATGTCCGTCACAGTGGAG-3', LAMA5-RV: 5'-TCATTCAGCGTGCCATCTC-3', COL4A1-FW: 5'-CCTCCCTGTACCTGGGCAGGC-3', COL4A1-RV: 5'-CCCTTGGGGCCAGGAAGACC-3', COL4A2-FW: 5'-CCAAGGAAGAGGTGGTGTGTCTG-3', COL4A2-RV: 5'-GGGCAGTGTGGGATGGAGAC-3', GAPDH-FW: 5'-ACCACTCCTCCACCTTTG-3', GAPDH-RV: 5'-CTCTTGTGCTCTTGCTGGG-3'. Every primer set was tested at different amplification cycles to ensure that any similarities in band intensity between the analyzed templates were not the result of reactions reaching the plateau phase. For LAMA1, 35 cycles of amplification were performed with denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, followed by a final extension step at 72°C for 7 minutes. For LAMA3 and LAMA5 amplification was performed as above, but for 34 cycles and annealing at 56°C. COL4A1 and COL4A2 were amplified for 35 cycles and 30 cycles with annealing at 64°C and 61°C, respectively. GAPDH was amplified for 24 cycles with annealing at 54°C. Each reaction was performed in a 50 µl volume containing 2.5 U HotstarTaq DNA polymerase (Qiagen, Valencia,

CA), 10×PCR buffer including MgCl₂ (Qiagen), 200 µM dNTP and 200 nM of forward and reverse primers. Control amplification was performed on RNA samples not subjected to reverse transcription to verify that no contaminating genomic DNA was present (data not shown). The PCR products were analysed by electrophoresis in 1.5% agarose gels.

Transplantation to nude mice

The parental cell line, HMT-3909S13 (Petersen et al., 1990; Rønnov-Jessen and Petersen, 1993), was tested for tumorigenicity by subcutaneous inoculation of 10⁶ cells in the right flank of 12 BALB/c-nu/nu mice. The tumorigenicity of the isolated myoepithelial subline, HMT-3909S16, was tested by transplantation of 10⁷ cells per mouse in 6 mice. The mice were observed for three months and tumor size was measured weekly.

Results

The functional unit in the normal human breast is the bilayered acinus with an inner layer of polarized luminal epithelial cells and an outer layer of myoepithelial cells separated from an interstitial collagen-I-rich stroma by an intact basement membrane. We had previously shown that in a laminin-rich reconstituted basement membrane (rBM) assay, normal luminal epithelial cells form an acinus with correct polarity even in the absence of myoepithelial cells (Petersen et al., 1992). We reasoned that in vivo myoepithelial cells most probably would carry on the task of directing the polarity of luminal epithelial cells. Accordingly we searched for an assay where this hypothesis could be tested.

Myoepithelial cells were specifically removed as previously described (Péchoux et al., 1999). The purified luminal epithelial cells were embedded in either collagen-I gels or in rBM to imitate the interstitial stroma and the basement membrane in vivo, respectively, and cultured for two weeks. This resulted in the formation of similar spherical, acinus-like structures, with a slightly smaller size in collagen-I gels compared with rBM (Fig. 1a,b). In addition, central lumina were observed only in rBM gels (Fig. 1c,d). Further characterization of the gels revealed additional fundamental differences in the structures of the acini in collagen-I gels and in rBM. Double staining for sialomucin and ESA showed an inside-out pattern in collagen-I gel, and a correct polarization (i.e. similar to the breast sections) in rBM (Fig. 2Aa,a'). The inside-out phenotype was further substantiated by double staining for sialomucin and the tight junction marker occludin (Fig. 2Ab,b'). Quantification of the number of correctly polarized profiles showed 0% in collagen-I gels (*n*=5 biopsies; 20 profiles per biopsy) compared with almost 100% in rBM. Furthermore, collagen-I gels, in contrast to rBM, failed to support either the basal targeting of β4-integrin (compare Fig. 2Ac and c') or the deposition of a continuous basement membrane as revealed by staining for type IV collagen (Fig. 2Ad,d') or laminin (not shown).

We then tested the hypothesis that the aberrant morphogenesis in collagen-I gel could be corrected by addition of myoepithelial cells. This indeed was the case since normal morphology and polarity could be dramatically rescued by addition of myoepithelial cells. On average, 70±8% of the acini in each experiment (*n*=5 biopsies) exhibited correct polarity, targeted β4-integrin to the basolateral surface and

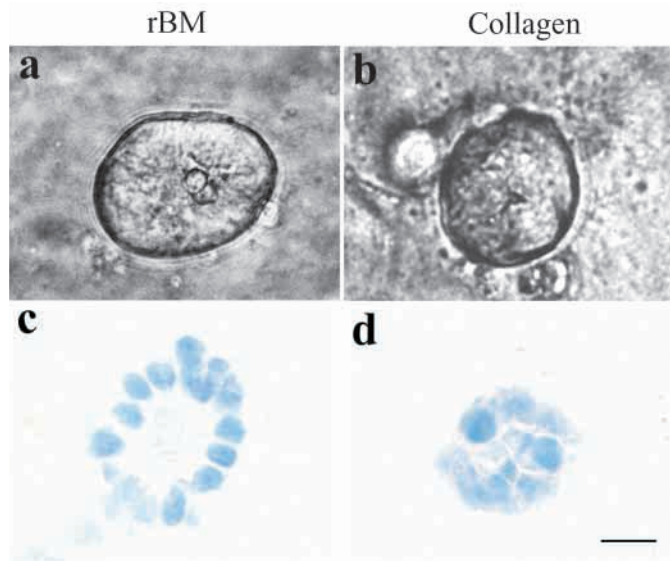


Fig. 1. Luminal epithelial cells form aberrant acini in collagen gels. Cells were embedded in either rBM (a,c) or hydrated collagen-I gels (b,d). (a,b) Phase-contrast microscopy revealed almost identical spherical structures in collagen-I and rBM albeit with no visible central lumen in spheres in collagen-I. (c,d) Haematoxylin staining of cryostat sections of the gels showed lumen formation only in rBM (bar, 25 μ m).

deposited basement membrane (Fig. 2Ba'',b'',c'',d''). This effect of myoepithelial cells on luminal epithelial spheres in collagen-I gels appears to be cell type specific since co-cultures of luminal epithelial cells with other breast cells (resident breast fibroblasts) or non-breast cells (osteosarcoma cells) did not lead to reversal of polarity or formation of double-layered acini (not shown).

Laminin can substitute for normal myoepithelial cells in reversing polarity in collagen-I gels

In collagen I gels, myoepithelial cells surrounded the epithelial cells and led to formation of double-layered acini similar to their positions in vivo (compare Fig. 3b and c) whereas, in rBM gels, myoepithelial and epithelial cells sorted out by themselves (Fig. 3a). Thus, among 20 randomly selected Thy-1-stained myoepithelial clusters in three different sections of EHS gels, we found no examples of inclusion of luminal epithelial cells forming an acinus or any evidence of central lumen formation. This indicated that under conditions where luminal epithelial cells were already correctly polarized, the myoepithelial cells were redundant. Since the laminin-rich matrix could substitute for myoepithelial cells, we tested whether laminin alone added to collagen-I gels could rescue polarity. The basement membrane in the human breast is characterized by the combined presence of three α -chains of laminin: α 1 in laminin-1, α 3 in

laminin-5, and α 5 in laminin 10/11 (Virtanen et al., 2000). These chains have been reported to be present in different stages of morphogenesis and cancer (Colognato and Yurchenco, 2000). Accordingly, we tested the effect of each of these laminins in the assay. Both laminin-1 and rBM (10% final concentration) could reverse polarity of luminal epithelial cells when added to collagen-I gels (Fig. 4). By contrast, neither affinity-purified laminin-5 (generous gift of Vito Quaranta and Jonathan Jones) nor affinity-purified laminin 10/11 (cat. #12163-010; Gibco BRL, Life Technologies A/S) (Ferletta and Ekblom, 1999) showed any evidence of a polarizing activity (Fig. 4). Similar data were obtained by use of a recombinant LG3-module of laminin-5 (kindly provided by Vito Quaranta; not shown).

These data would predict that (1) luminal epithelial cells do not make sufficient laminin-1 to allow them to polarize in collagen-I gels, and (2) that normal myoepithelial cells are the

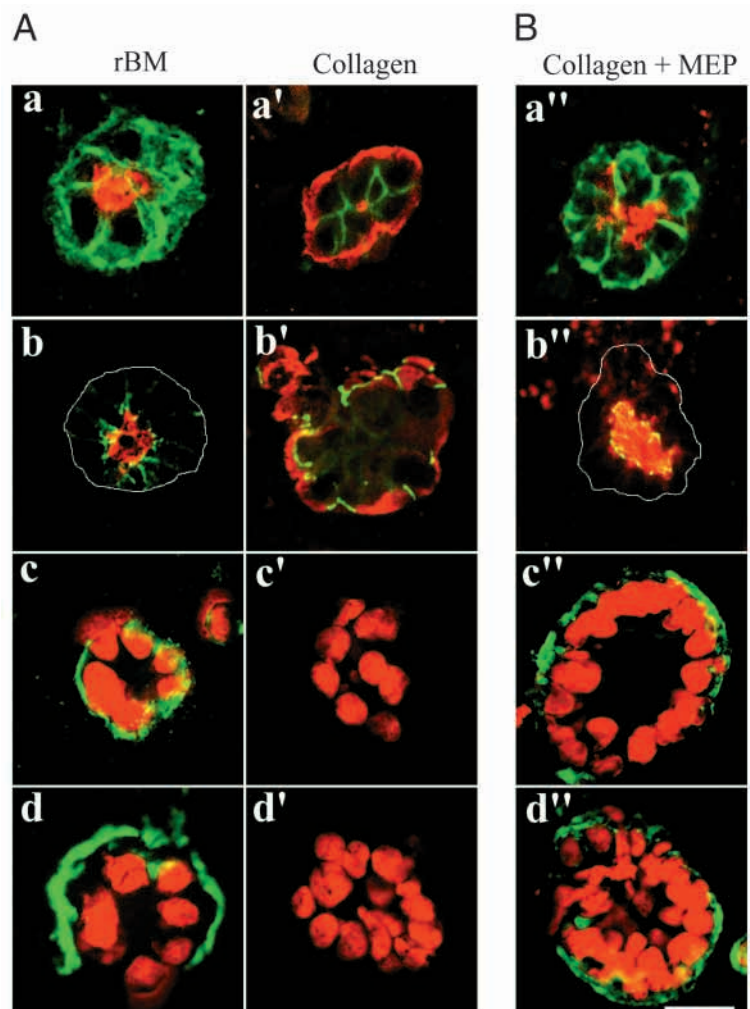
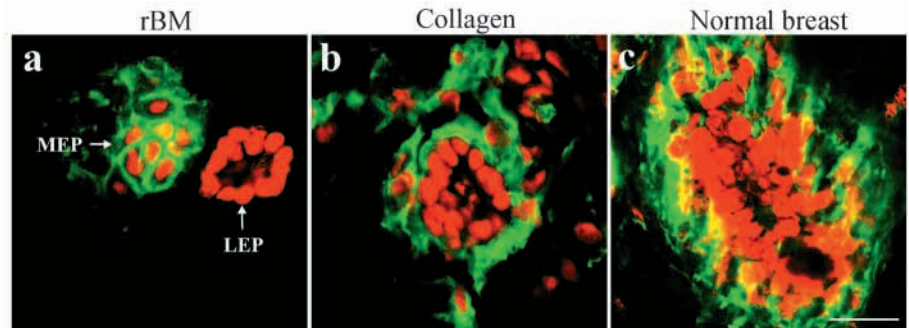


Fig. 2. (A) Luminal cells make inside-out acini in collagen. Luminal cells were double-stained either for (a) sialomucin (red) and ESA (green); (b) sialomucin (red) and occludin (green); (c) nuclear stain (red) and β 4-integrin (green); or (d) nuclear stain (red) and type IV collagen (green). Spheres in collagen-I gel (a',b') exhibit reversed polarity compared with cells in rBM (a,b), do not target β 4-integrin basolaterally (compare c and c') and fail to deposit a basement membrane (compare d and d'). (B) Reversal of inside-out acini by addition of myoepithelial cells. In the presence of myoepithelial cells (a''-d''), the polarity is corrected as is the endogenous BM deposition and integrin targeting. (bar, 25 μ m).

Fig. 3. Luminal epithelial cells and myoepithelial cells sort themselves out inside rBM gels but form bilayered acini inside collagen gels. The spatial organization of myoepithelial cells in relation to luminal epithelial acinar backbones was analyzed in rBM (a) and hydrated collagen-I gel (b) and compared to that in normal breast (c). Gels were doublestained for Thy-1 to demonstrate myoepithelial cells (green) and propidium iodide to demonstrate nuclei (red). Note that whereas myoepithelial cells segregate from luminal epithelial acini in rBM, in vivo-like double-layered structures are formed in the collagen-I based acinus assay (bar, 25 μ m).



source of the polarizing principle through synthesis of laminin-1. To test these predictions, the synthesis of basement membrane components by normal luminal cells and myoepithelial cells was evaluated using RT-PCR. A tumor cell line that did not synthesize laminin-1 (not shown) was used as negative control. Luminal epithelial cells made little or no laminin α 1 chain either in collagen-I or in rBM. By contrast, myoepithelial cells expressed copious amounts of laminin α 1 (Fig. 5). Both cell types expressed α 3-chain and the α 5-chain of laminin in addition to α 1- and α 2-chains of type IV collagen. Furthermore, immunostaining of sorted luminal epithelial- and myoepithelial cells within (mouse) rBM with antibodies specific to (human) 400 kDa α 1-, the 165 kDa α 3-, and the 380 kDa α 5-chain (Rousselle et al., 1991; Virtanen et al., 2000), revealed that the critical difference could be narrowed down to the lack of deposition of α 1-chain-containing laminin by luminal epithelial cells (Fig. 6). A similar pattern was obtained when established normal cell lines derived from myoepithelial and luminal epithelial cell origin were examined (not shown). Since myoepithelial cells and pure laminin-1, but not laminin-5 or laminin-10/11, correct luminal epithelial cell polarity, and because myoepithelial cells

synthesize laminin-1 and luminal cells do not, we conclude that laminin-1 is important for signalling and crosstalk between myoepithelial and luminal epithelial cells in vivo (Rousselle et al., 1991; Colognato and Yurchenco, 2000; Virtanen et al., 2000).

Cancer-derived myoepithelial cells share many characteristics of normal myoepithelial cells but fail to reorient inside-out acini

One of the hallmarks of breast cancer is loss of polarity and organization of epithelial cells. It is also known that many breast tumors lack myoepithelial cells (Rudland, 1987). However, even in the cases where myoepithelial markers have been detected, luminal cells remain largely disorganized (Wetzels et al., 1989; Malzahn et al., 1998). We asked whether these tumor myoepithelial cells lacked the ability to signal to luminal cells for polarity, and whether this was due to their inability to synthesize laminin-1.

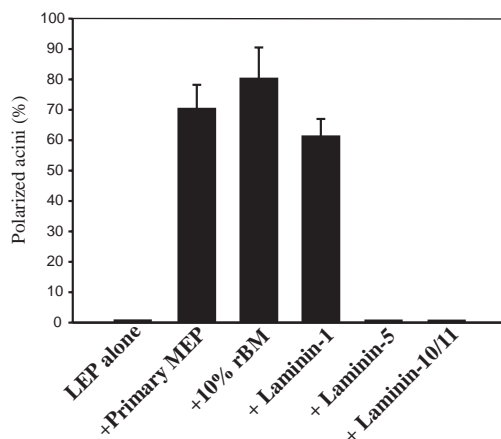


Fig. 4. Only laminin-1 and not laminin-5 and -10/11 can correctly polarize acini in collagen gels. Luminal epithelial cells without myoepithelial (LEP alone) had no correctly polarized acini. Addition of 10% rBM or pure laminin-1 reversed polarity to the same degree as normal-derived myoepithelial cells (MEP). Neither affinity purified laminin-5 nor laminin-10/11 possessed the ability to revert acinus polarity.

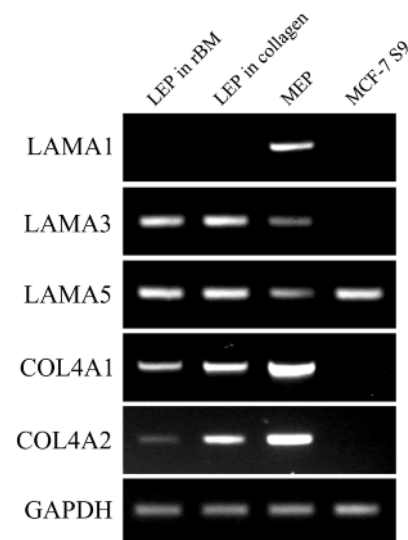


Fig. 5. LAMA-1 chain is expressed only in myoepithelial cells. RT-PCR analysis of laminin α 1 (LAMA1), laminin α 3 (LAMA3), laminin α 5 (LAMA5), type IV collagen α 1 (COL4A1) and type IV collagen α 2 (COL4A2) in luminal epithelial cells in rBM and collagen-I gel and in myoepithelial cells and MCF-7 cells. GAPDH is used as an internal control. Note that the only laminin chain that is not expressed in the pure luminal epithelial cultures is the α 1-chain.

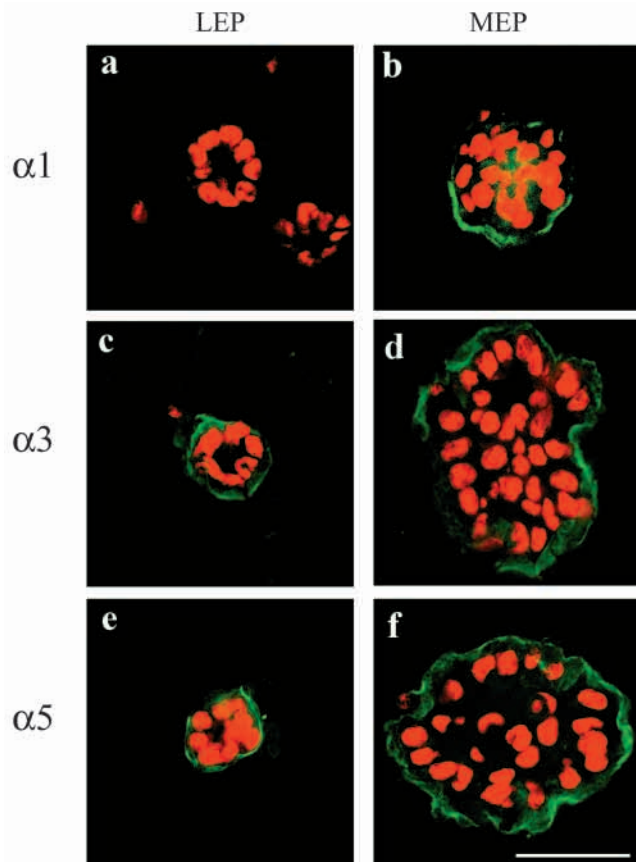


Fig. 6. Luminal epithelial cells and myoepithelial cells can deposit both $\alpha 3$ -chain and $\alpha 5$ -chain of laminin but only myoepithelial cells can deposit laminin- $\alpha 1$ chain. Luminal epithelial cells (a,c,e) and myoepithelial cells (b,d,f) embedded in rBM were stained for laminin $\alpha 1$ (a,b; green), laminin $\alpha 3$ (c,d; green) and laminin $\alpha 5$ (e,f; green). Both luminal epithelial cells and myoepithelial cells deposit a basement membrane except for the lack of $\alpha 1$ -chain deposition by the former. Nuclear stain, red. (bar, 25 μ m).

Since it is not simple to isolate myoepithelial cells from breast cancers, we initially used a breast cancer cell line (HMT-3909S13) previously established in our laboratory (Petersen et al., 1990; Rønnev-Jessen and Petersen, 1993) that produces myoepithelial cells at the stromal-epithelial junction in tumors generated in nude mice. These tumors resemble biphasic human breast carcinomas as shown by double-staining with human specific antibodies to vimentin and keratin (not shown) (Malzahn et al., 1998). We purified the myoepithelial cells from HMT-3909S13 by immunomagnetic sorting using $\alpha 1$ -integrin antibody (see Materials and Methods). These cancer-derived myoepithelial cells, referred to as HMT-3909S16, further resemble normal myoepithelial cells by a number of criteria: they emerge from the luminal epithelial lineage (Péchoux et al., 1999) as shown by clonal culture; they are myoepithelial restricted in their differentiation repertoire once isolated from the cancer cells; they coordinately express vimentin, keratins and α -smooth muscle actin (Fig. 7Aa,b), and they are non-tumorigenic in nude mice even after injection of ten times as many cells as were sufficient for tumor-take with the parental line (0/6 mice compared with 11/12 mice). Collectively,

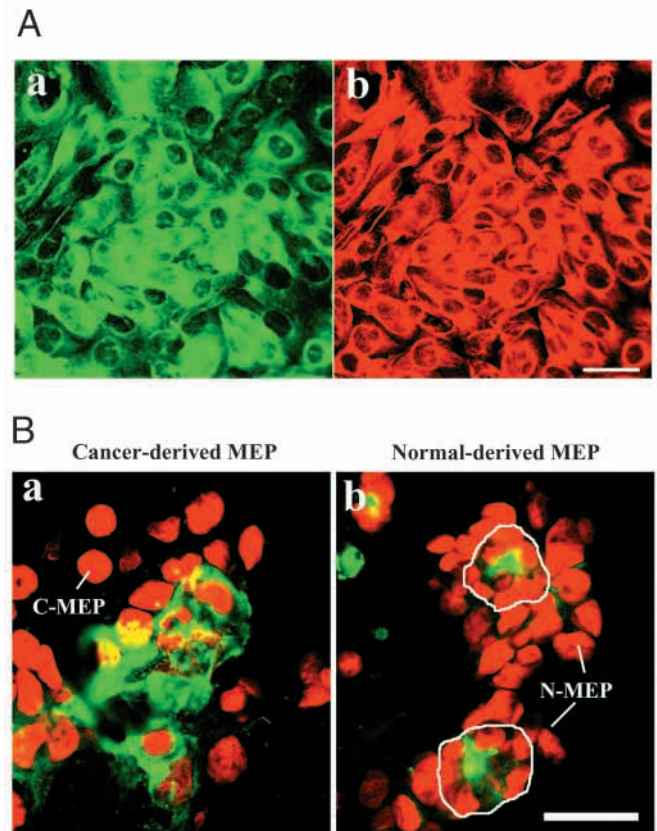


Fig. 7. Cancer-derived myoepithelial cells share many characteristics of normal myoepithelial cells but fail to reorient inside-out acini. (A) Isolation of cancer-derived myoepithelial cells expressing typical myoepithelial markers. Purified cancer-derived myoepithelial cells (a,b) stained with α -smooth muscle actin (green) and keratins (red) to document the concurrent myo- and epithelial phenotypes (bar, 50 μ m). (B) Lack of reversal of inside-out acini by cancer-derived myoepithelial cells. Sections of (a) the acinus assay embedded with the cancer-derived myoepithelial cell line and (b) an immortalized, normal-derived myoepithelial cell line. The sections are double stained for the apical marker sialomucin (green) and the nuclear stain propidium iodide (red). Note the lack of polarization with the cancer myoepithelial cells (C-MEP) and the correctly polarized lumina (encircled) with the immortalized myoepithelial cells (N-MEP) (bar, 25 μ m).

therefore, these cells qualify as myoepithelial cells. Nevertheless, they completely failed to correct polarity of luminal cells in collagen-I gels even at cell numbers ten times higher than those sufficient for normal myoepithelial cells to elicit this function (Fig. 7Ba). Since these cells were immortal, and the normal myoepithelial cells used in our assay were not, we asked whether the inability to correct polarity was related to immortalization. We therefore also immortalized purified normal myoepithelial cells with E6/E7, and cultured them for a similar number of passages (40) as the cancer-derived myoepithelial cells. Co-culture of the normal-derived myoepithelial cell line with luminal epithelial cells in the collagen I assay resulted in correct polarization in $63 \pm 5\%$ of the acini (Fig. 7Bb, Fig. 9), in contrast to zero percent (Fig. 7Ba, Fig. 9) when the cancer-derived myoepithelial cell line was used.

Table 1. Myoepithelial differentiation in normal and cancer-derived myoepithelial cells

Differentiated trait	Immortalized MEP cell line	Cancer-derived MEP cell line
Laminin-1*	+	–
Laminin-5	+	+
Laminin-10/11	+	–/+‡
α -sm actin	+	+
Keratins	+	+
Vimentin	+	+
α 1-integrin	+	+
α 3-integrin	+	+

*All markers listed are positive in primary cultured myoepithelial cells.

‡Positive cells in the presence of serum.

Cancer-derived myoepithelial cells fail to revert inside-out acini unless they synthesize laminin-1

To test the hypothesis that the critical difference between immortal myoepithelial cells isolated from normal and neoplastic breast tissue was their ability to synthesize laminin, we stained the cells for a number of myoepithelial differentiation markers (Table 1). The major difference in the markers tested was clearly the ability to make laminin-1. This difference was further confirmed by RT-PCR (Fig. 8). To substantiate the significance of these observations further, we purified myoepithelial cells from 3 primary carcinomas, being well aware that these could contain cells from residual benign ducts. Nevertheless, in two of the three preparations, the carcinoma-derived/-associated myoepithelial cells behaved in a manner similar to the established myoepithelial cell line (Fig. 9). The one cancer-derived/-associated myoepithelial cell sample which could polarize the luminal epithelial cells also contained myoepithelial cells staining for laminin-1 (Fig. 9).

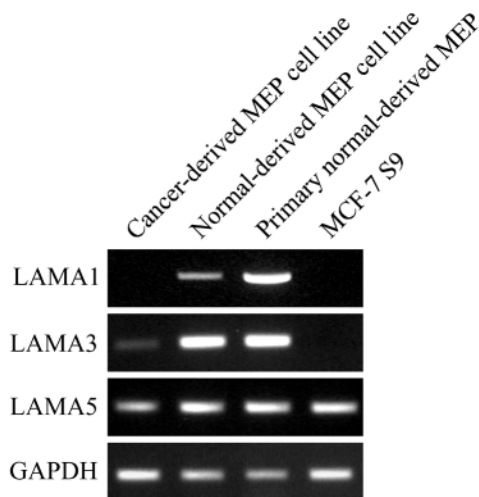


Fig. 8. The cancer-derived myoepithelial cell line lacks the expression of laminin- α 1 chain only. RT-PCR on RNA extracted from cancer-derived myoepithelial cell line, immortalized, normal-derived myoepithelial cells (MEP), primary cultured MEP and MCF-7 S9 cells. The panel shows α 1-chain (LAMA1), α 3-chain (LAMA3), α 5-chain (LAMA5) and GAPDH as an internal control. While the α 3 mRNA was low in the cancer-derived MEP cell line, the α 1-chain was the only differentially expressed chain between normal-derived and cancer-derived myoepithelial cells.

Furthermore, a basement membrane, albeit fragmented, was deposited, and β 4 integrin was targeted to the basal cell surface (Fig. 9). To test whether the data obtained with tumor-derived myoepithelial cells in culture was indeed representative of tumor tissue, we further stained 12 infiltrating ductal breast carcinomas with laminin-1. Seven carcinomas were completely negative for laminin-1. The other five carcinomas including one carcinoma in situ showed foci of fragmented basement membrane staining which was clearly less pronounced than in normal breast tissue (Fig. 10). Double staining for either of the myoepithelial markers maspin, keratin K17 or keratin-associated BG3C8 (not shown) and laminin-1 revealed that tumor-associated myoepithelial cells were either negative or weakly positive for laminin-1 (Fig. 11). Whenever residual normal breast tissue was present in the carcinomas (2/12), a strong staining for laminin-1 was recorded in connection with normal cells. We conclude that, even though myoepithelial cells of tumors exhibit many traits in common with normal myoepithelial cells, it is the production of laminin-1 that determines their capacity for heterotypic crosstalk.

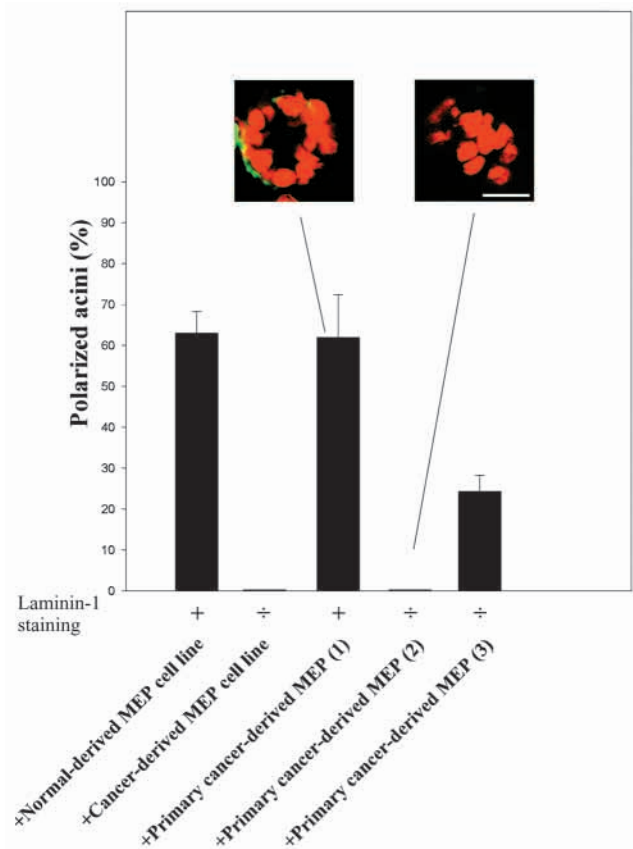


Fig. 9. Only one of four cancer-derived myoepithelial cells can completely reverse acinus polarity in collagen gels. Frequency of correctly polarized acini in sections of collagen-I gels. Addition of cancer-derived myoepithelial cells from four different sources failed completely to revert acini in two instances, had a partial impact on reversion in one instance, and could revert in the fourth case. Inserts show staining for β 4-integrin (green) counterstained with propidium iodide (red). Data represent mean (\pm s.e.m.) except for the primary cancer-derived MEP, which are means of triplicate determinations (\pm s.d.). (bar, 20 μ m).

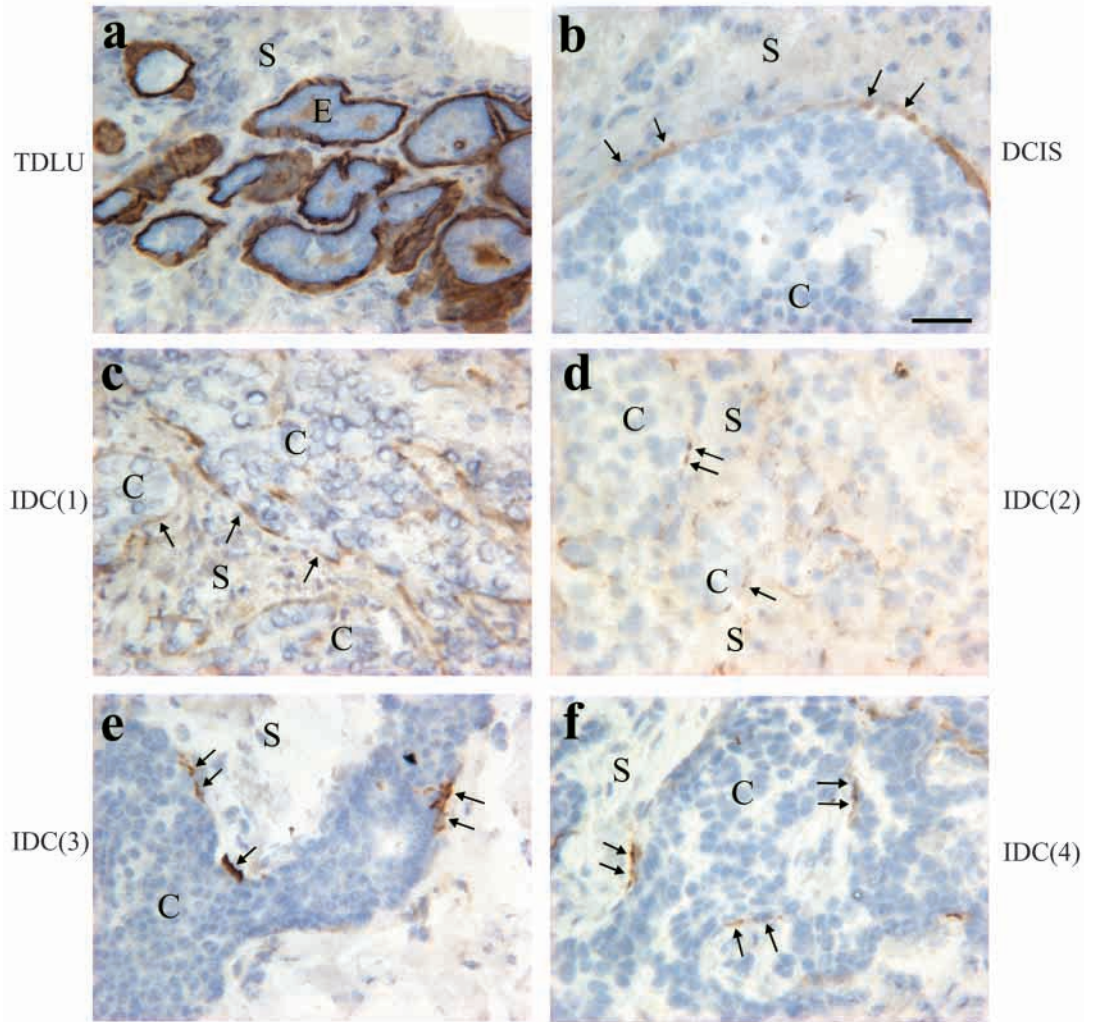


Fig. 10. Reduced staining of laminin-1 in human breast cancer. Cryostat sections of terminal duct lobular unit (TDLU) (a), a ductal carcinoma in situ (DCIS) (b) and four different infiltrating ductal breast carcinomas (IDC) (c-f) stained with immunoperoxidase for laminin-1 and counterstained with hematoxylin. In the TDLU a strongly stained, continuous basement membrane is delineated at the stromal (S)-epithelial (E) interface (a). In carcinomas, laminin-1 staining is localized to the stromal (S)-cancer epithelial (C) interface, but is discontinuous and less pronounced. (bar, 25 μ m).

Discussion

In the present study we demonstrate that myoepithelial cells in the normal human breast function to maintain correct polarity of the cellular axis of luminal epithelial cells through their ability to synthesize laminin-1. We also present evidence that a majority of the tumor-derived/-associated myoepithelial cells tested, even when they retain an extensive myoepithelial differentiation program, fail to correct luminal epithelial polarity because they have lost their ability to make laminin-1.

The function of normal myoepithelial cells

The cells of the normal human breast have been assayed previously in collagen-I gels, but these preparations included myoepithelial cells (Foster et al., 1983; Hamamoto et al., 1988). Thus, functional analysis of pure normal epithelial or myoepithelial cells within the context of breast morphogenesis has awaited a sufficiently sophisticated technology to separate the different cell populations with immunomagnetic antibodies (Clarke et al., 1994; Gomm et al., 1995; Péchoux et al., 1999). Nevertheless, that a major function of myoepithelial cells is to manufacture basement membrane material was inferred from studies of myoepithelial cell lines (Warburton et al., 1981; Kedeshian et al., 1998). Laminin-1 was important for

functional differentiation of mouse mammary cells grown in collagen-I gels (Streuli et al., 1991) and for polarization of intestinal, kidney and breast epithelial cells in monolayer culture (Klein et al., 1988; De Arcangelis et al., 1996; Slade et al., 1999). Because specific antibodies to human laminin-1 have not been readily available, it has been difficult to correlate its presence with an observed activity *in vivo* or in collagen cultures. One available antibody which was assumed to react with the α 1-chain of laminin-1 (Engvall et al., 1986; Engvall et al., 1990), has more recently been shown to react specifically with the α 5-chain of laminin 10/11 (Tiger et al., 1997). Thus some of the literature using this antibody needs to be re-evaluated because the α 5-chain has a wider distribution than the α 1-chain (Virtanen et al., 2000). Only recently, a monoclonal antibody that is specific for the α 1-chain of laminin became available. Thus it is now possible to analyze the human tissues to complement the results with *in situ* hybridizations and those of immunostainings of mouse tissues (Virtanen et al., 2000). In the present study we used this antibody (a kind gift of I. Virtanen) in parallel with our RT-PCR reactions.

Previous studies have suggested that the expression pattern of the α 1-chain of laminin-1 is largely restricted to the fetal tissues (Tunggal et al., 2000) and that the α 1-chain is replaced

by the $\alpha 5$ -chain of laminin 10/11 in the adult (Ekblom et al., 1998; Tunggal et al., 2000). However, in the adult human breast, $\alpha 1$ -chain remains expressed together with $\alpha 5$ -chain (Virtanen et al., 2000). We suggest this is because the breast continues to undergo developmental-like changes in the adult during menstrual cycles, puberty, pregnancy and menopause. As such, it is a continuously developing organ.

Another member of the laminin family expressed in the human breast basement membrane is the $\alpha 3$ -chain of laminin-5 (Martin et al., 1998). Laminin 5 expression has been associated with cell differentiation during tooth development (Salmivirta and Ekblom, 1998). Compatible with a differentiating function, laminin 5 has been suggested as a potential tumor suppressor in the human breast (Martin et al., 1998). In the present study we identify myoepithelial cells as the only source of $\alpha 1$ chain while both $\alpha 3$ - and $\alpha 5$ -chains are expressed by luminal epithelial cells also.

How different tissues achieve polarity is undoubtedly governed by some broad, universal rules. However, the specific details would most certainly have to be tissue specific. Thus, in an epithelial kidney cell line (MDCK), clonal cells were reported to polarize in collagen-I gels independently of the deposition of laminin-1, and it was concluded that basal lamina formation was not required for maintenance of structural and functional polarity (Ecay and Valentich, 1992). This may, however, pertain only to certain cloned cells (see Note added in proof). Both kidney and thyroid epithelial cells can undergo polarity reversal, as is shown here for breast luminal epithelial cells, except the shift occurs between suspension cultures and embedding inside collagen gels (Gumbiner, 1990). Moreover, even among different cell lines of the same tissue, different results have been reported from different laboratories. Thus caution needs to be exercised when the information from cell lines is extrapolated to the parental tissue. For example, the 'normal' murine mammary epithelial cell line, NMuMG, cultured inside attached collagen gels, formed polarized acini in the absence of added laminin and with little or no apparent endogenous laminin deposition (Soriano et al., 1995). The pancreatic cell line S2-013, which forms correctly polarized acinar structures in both collagen gel and in rBM, deposits an endogenous basement membrane only in rBM (Yamanari et al., 1994). Moreover, we have shown previously that the human breast carcinoma cell line used in the present study as a negative control for the formation of basement

membrane and expression of laminin-1, can polarize on tissue culture plastic without a laminin coating and can form a complete set of sealed tight junctions and a specialized apical cell membrane domain with microvilli (van Deurs et al., 1987). Therefore, the reverse polarity of normal human primary breast epithelial cells in collagen-I gel

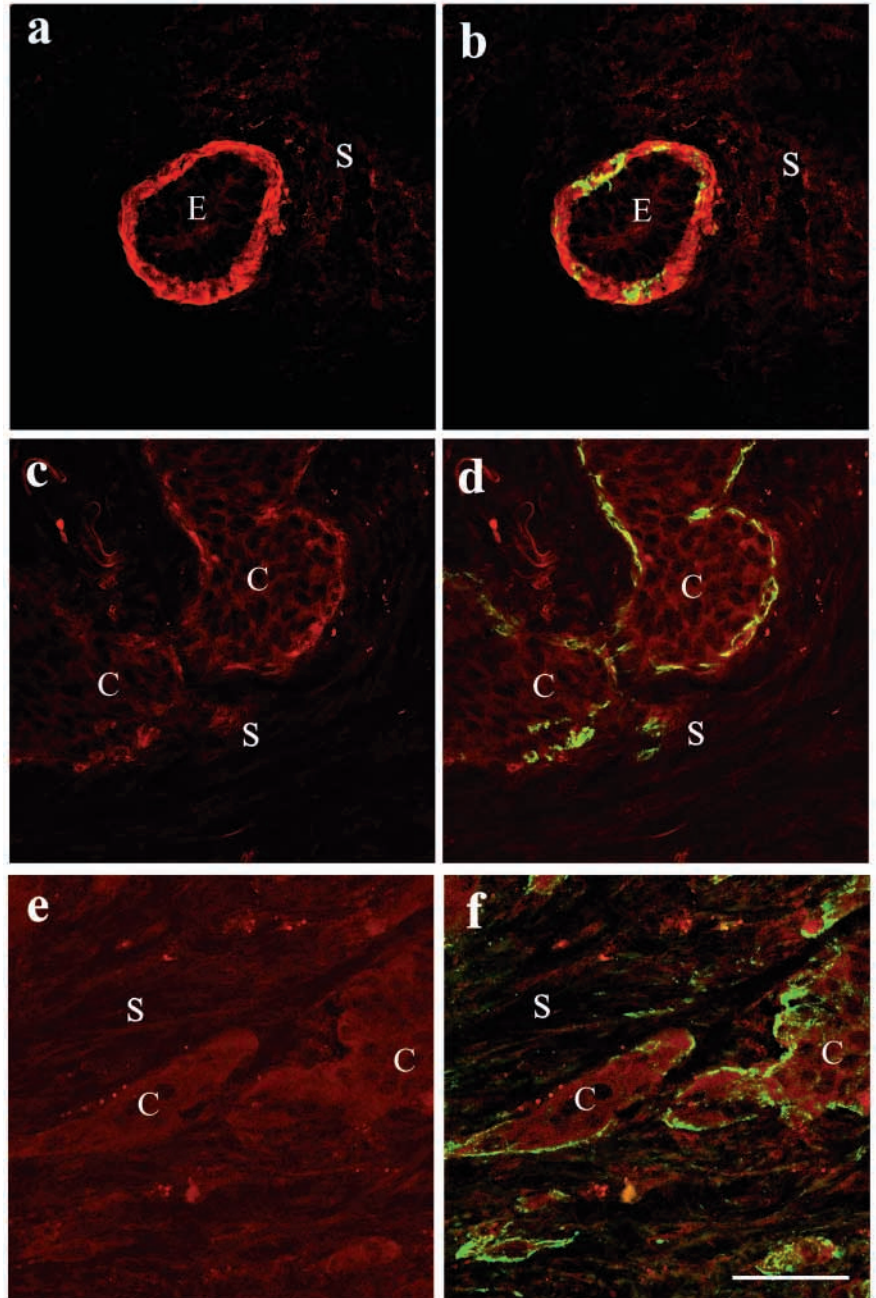


Fig. 11. Absence of, and reduced staining of laminin-1 in tumor-associated myoepithelial cells. Double-labeling immunofluorescence of a normal acinus in a TDLU (a,b) and two different carcinomas (c,d and e,f) to demonstrate laminin-1 in red (a,c,e) and in a coexpression with myoepithelial cells in green (b,d,f) as stained with keratin 17 (b,d) or maspin (f). The bottom panels illustrate the presence of myoepithelial cells at the stromal (S)-cancer epithelial (C) interface in total absence of laminin-1 staining. The middle panels show infiltrating ductal carcinoma with tumor-associated myoepithelial cells expressing a variable but generally weak staining for laminin-1 compared with a normal acinus in the top row. (bar, 50 μ m).

and their response to myoepithelial cells could not have been easily inferred from studies on other tissues or cell lines. Finally, correct polarization in collagen gel has been reported for epithelial cells of the intestine (Kirkland, 1988), lung (Sugihara et al., 1992), endometrium (Kirk and Alvarez, 1986), gall bladder (Mori and Miyazaki, 2000) and several other tissues. In fact, we have not been able to find another example of epithelial cells with an inside-out pattern of polarity in collagen gel, suggesting that the normal human breast gland with its well developed layer of myoepithelial cells may be unique in this behaviour. Therefore, breast luminal and myoepithelial cells together may indeed be the 'unit of polarity' in breast.

Some cancer-derived myoepithelial cells are deficient in their ability to reverse luminal polarity

It is generally believed that breast cancers lack myoepithelial cells (Li et al., 1998). While certain biphasic or bimodal carcinomas manufacture cells with a partial myoepithelial differentiation program, this does not confer a better prognosis for the patients (Malzahn et al., 1998). These data could argue against the postulate that myoepithelial cells can act as tumor suppressors. The finding of functional defects in cancer-derived myoepithelial cell lines and two of the primary cell lines may provide an explanation for the above observations in vivo. In some breast carcinomas, neoplastic myoepithelial cells are present and appear to be functional. This is the case with the adenoid cystic carcinoma, which has a favorable prognosis and does not metastasize to distant sites (Kasami et al., 1998). In these carcinomas, laminin is deposited, and the neoplastic luminal epithelial cells polarize correctly (Kasami et al., 1998). Nevertheless, in the light of the above discussion on specificity of the existing laminin antibodies, the deposition of laminin-1 needs to be confirmed using the new specific antibodies. Only very recently, the staining pattern of laminin-1 using the same antibody as used in the present study (Clone 161 EB7) was conducted on a panel of tumors from a number of tissues (Määttä et al., 2001). It was concluded that laminin-1 was generally absent from invasive breast carcinomas and that it was strongly expressed around normal breast epithelial structures (Maatta et al., 2001). This is compatible with the studies reported here. We show additionally that whenever actually present focally, the laminin-1 staining is weak and fragmented, and that it may colocalise with the expression of other markers of myoepithelial differentiation. Of the three primary myoepithelial cell samples isolated from carcinomas, one was able to almost completely revert the normal luminal cells and one had partial activity. Although this could be interpreted in favour of different degrees of 'contamination' of tumor tissue, we have additional data to suggest that, in some other respects, these cells did not behave exactly like normal-derived myoepithelial cells in our assay. However, regardless of the source of myoepithelial cells or their otherwise aberrant behaviours (Jones et al., 1992), correlation between the expression of laminin-1 and reversal of polarity was essentially complete.

Currently, the data for the frequency of myoepithelial differentiation in breast carcinomas are contradictory, because they vary with the marker tested. However, in the very common, grade I infiltrating ductal carcinoma, which has a

favorable prognosis and is often polarized correctly (Lundin et al., 2001), a fragmented basement membrane has been described in 75-90% of the cases (Albrechtsen et al., 1981; Gusterson et al., 1982). At the ultrastructural level, myoepithelial-like cells have also been reported in grade I carcinomas (Hayashi et al., 1984). Finally, several reports have correlated the ultrastructural or immunoreactive presence of myoepithelial-like cells with an ultrastructurally or immunocytochemically defined basement membrane (Gould et al., 1975; Wetzels et al., 1989; Senzaki et al., 1992).

The double-layered acinus

The ultimate unit of function in any organ is clearly the organ itself. In the breast, an important component of this functional unit is the double-layered acinus composed of luminal and myoepithelial cells. While this is not strictly equivalent to the breast gland, it nevertheless captures an essential component of the functional entity of the breast. In this report, we have achieved both an assay for formation of the double-layered acinus and have unraveled an important underlying mechanism for its formation. We propose that the inability to maintain the polarized double-layered structure in breast tumors is a result of aberrant laminin-1 production by myoepithelial cells. This may be a fundamental reason behind the poor prognosis in some breast cancers. We further propose that both the prognosis and treatment of breast cancer should consider this structural and functional unit as a target.

Note added in proof

Since the submission of this manuscript on January 12, 2001 two papers have appeared both in the same issue of *Nature Cell Biology* that bear on our current report: O'Brien, L. E. et al. Rac1 orientates epithelial apical polarity through effects on basolateral laminin assembly. *Nat. Cell Biol.* **3**, 831-838, 2001, and Runswick et al. Desmosomal adhesion regulates epithelial morphogenesis and cell positioning. *Nat. Cell Biol.* **3**, 823-830, 2001. O'Brien et al. show that apical polarity in MDCK epithelial cysts requires both laminin and Rac1. Runswick et al. demonstrate that more than just cell-ECM interactions contribute to the double-layered acinus formation.

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